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STAIN TECHNOLOGY

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Soil Bacteriologist, N. Y. Agr. Exp. Station

Consulting Editors

CHEMISTRY: W. C. HOLMES Color Lab., U. S. Dept. of Agriculture

ZOOLOGY: S. I. KORNHAUSER School of Medicine, Univ. of Louisville

PATHOLOGY: F. B. MALLORY Boston City Hospital

BOTANY: W. R. TAYLOR Univ. of Michigan

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STAIN TECHNOLOGY

VOLUME V OCTOBER, 1930 NUMBER 4

THE HISTORY OF STAINING

THE DEVELOPMENT OF CYTOLOGICAL STAINING

S. I. KORNHAUSER, *University of Louisville Medical School, Louisville, Ky.*

Perhaps no other period has contributed more to our knowledge of the cell than the period 1875–1895. During these years most fundamental cytological phenomena were seen and described. Mitosis, maturation and fertilization, the great cornerstones of cytology, were firmly laid by the remarkable researches of Flemming, Strasburger, Van Beneden, Oscar and Richard Hertwig, Boveri and many others. Upon these researches experimental cytology developed and the significance of the morphological phenomena to inheritance and development was pointed out by such masters as W. Roux, Weismann, O. Hertwig, Boveri and E. B. Wilson.

From 1875 to 1895 there was a great advance in microscopy along all its lines. The microscope itself was greatly improved. The development of homogeneous immersion objectives, of better substage condensers and later apochromatic objectives, largely thru the genius of Ernst Abbé, all contributed to making possible the discoveries of many minute details of cell structure and cell change.

The proper fixation of tissues had to be worked out largely by the "cut and try" method. At first it was thought that fixation and staining might be made one process and thus we find picro-carmin, acetic-carmin and acetic-methyl green much in use in early cytological researches.

But as the necessity for accurate thin sections appeared, fixation became a separate process. This step had to be followed by infiltrating, embedding, cutting and mounting the sections. Thus the development of celloidin and paraffin embedding, the invention of new types of microtomes and material to affix sections, paved the way for more elaborate and exact staining of sections on the slide. This latter point is the chief topic of this paper. It is perhaps significant that with advances in staining came the general use of resinous mounting media for sections, Canada balsam or damar, which replaced, in large part, glycerin or clove oil for permanent preparations.

The technical journals and manuals of microscopy of 1870 dealt very largely with optical problems and accessories for the microscope, and comparatively small space was given to methods of preparation of material. We find that the injection of blood vessels for microscopical examination was well worked out: silver salts were used for bringing out intercellular spaces, osmic acid was advocated for blackening fat and myelin, whereas practically the only stain was carmin.

Silver was used as early as 1854 by Flinzer in his study of the cornea. Largely thru the work of Ranvier (1868), Golgi (1873) and more recently Cajal, the use of silver in neurological work has been of increasing importance and has also lately been used much by cytologists in the study of the Golgi apparatus. Gold salts also are of great value in neurological technic and have been in use since employed by Cohnheim (1866) for the study of peripheral nerve endings. Perhaps not less important to neurologists is a third rare metal, osmium. Max Schultze employed osmic acid as early as 1865 in his classical studies on protoplasm. It has served cytologist, neurologist and histologist well, and in spite of its irritating fumes has remained the valued friend of the microscopist. The cytologist values it chiefly as an ingredient of several important fixing fluids, whereas the neurologist finds its blackening of myelin an important property. Lately the cytologists interested in cell inclusion have come to use it a great deal in the study of the cyto-reticulum, which it can be made to blacken by proper fixation.

Of the natural dyes early used by cytologists none was more esteemed than carmin. Even today we would be loathe to part with this substance. It is still valuable to the embryologist as a bulk stain of great permanence, and to the cytologist as a medium for the rapid staining and examination of chromosomes in fresh material. Masterpieces of cytological research were done using carmin as the principal stain. Flemming (1874), who is considered the father of modern technic, used carmin in his studies on the egg of Anodonta. Oscar Hertwig (1876) in his classical research on the fertilization of the echinoderm ovum also employed carmin, as did Van Beneden (1883) in his most remarkable study of the maturation, fertilization and cleavage of Ascaris. Boveri (1887, 1890) in his first three important cell studies used carmin almost exclusively and found that the acetic carmin mixture of Anton Schneider (1880) was very valuable. If we turn to the first edition of Lee's Vade-Mecum 1885 we find on page 41 the following, "Carmin which is probably the most valuable and certainly is the most widely employed of histological

coloring agents was first proposed as an aid in examining animal tissues by Gerlach 1858, since which time, notwithstanding the discovery of numerous other substances that have proved most useful in many kinds of research, it has held its place with a firmness that shows few signs of yielding as a staining agent par excellence." To Paul Mayer, who contributed so much to many phases of microscopic technic, we owe our exact knowledge of the nature of carmin and better formulae for carmin stains than existed up to his researches of the eighties and nineties; (see earlier paper of this series, Stain Techn. **3**, 18–9, 1928).

Turning from carmin we find that hematoxylin is the only other natural dye early in use to be retained in full popularity by the microscopist.

For general histological work Delafield's and Ehrlich's hematoxylin solutions are invaluable; for special purposes one should mention Weigert's method (1884) for myelin and Mallory's phosphomolybdic (1891) and phospho-tungstic acid (1897) hematoxylins, which are certainly valuable in every histological laboratory. For the cytologist, however, iron hematoxylin holds the foreground. Due to the distinctness with which it brings out cell structures and to its permanency it is very popular. Benda (1886) is to be regarded as the originator of the iron hematoxylin method in that he used ferric ammonium sulfate as a mordant previous to employing a ripened hematoxylin solution. Martin Heidenhain's method (1892) however, has come into general use earning its popularity thru its own value. Perhaps the only drawback to this stain is that it brings out too many cell structures in black or dark grey, as for instance, chromosomes, centrosomes, chondriosomes and yolk granules. It is almost always necessary therefore for the cytologist to use the more selective anilin dyes to check up iron hematoxylin preparations.

Many natural dyes were used by early microscopists, among which were blueberry juice, red-cabbage juice, indigo, alizarin, and purpurin. The last three are now manufactured synthetically and of these the cytologist employs only alizarin in the form of sodium-alizarin-sulfonate. This is used in the Benda mitochondrial technic (1901) staining the chromosomes a weak brown.

We now turn to the largest and most valuable group of dyes, the anilins, which were used so effectively in early cytological research by Walter Flemming, whose name is intimately connected with the staining and fixation of cells. In his studies in the early seventies on connective tissue and on the ovum of Anodonta, Flemming used relatively simple technic: carmin-glycerin preparations of bichromate

or picric acid fixed material cut by the freezing method or by his glycerin soap method. A few years later, however, he became acquainted with the staining method used by Hermann (1875), and this undoubtedly stimulated Flemming in his pursuit of the anilin dyes as an aid to the microscopist. Hermann had used fuchsin as a regressive stain, that is, he overstained his sections and then decolorized them in alcohol and clove oil and mounted in balsam.[1] In Flemming's paper (1877) on the characteristics of the cell nucleus we find him using Hermann's method. In this paper he showed the chromatin network and the karyosomes but no mitoses were pictured. It seems also that in these first papers on the cell Flemming used clove oil to mount his sections and this decolorized them much to his disappointment. In 1879 we find his first extensive paper on mitosis in which he showed the details of the changes of the chromatin in most beautiful manner. His slides were stained with safranin, which was a great favorite of Flemming. He too was interested in the fixation of cells and entered into considerable discussion with his contemporaries over the relative value of chromic acid which he advocated as against bichromate of potash then in more general use.

About this same time Max Flesch (1879) made a further advance in the fixation of cells. He brought out his mixture of chromic and osmic acids in the same volume in which Flemming's article above cited appeared. He also described methods for embedding tissues in tallow, and balsam as a mounting medium for sections. Flesch's mixture was used by Flemming in subsequent work and praised by him greatly. Later Flemming (1882) improved Flesch's fixing fluid by the addition of acetic acid and this chrom-osmo-acetic mixture was *Flemming's fluid, weak*, an excellent cytological reagent, but now largely supplanted by *Flemming's fluid, strong*, in which the concentration of the acids is greatly increased. Even today no fixing fluid has been discovered which gives a more faithful picture of cell structure. It remains the most esteemed cytological fixing agent the world over. Thus Flemming was always willing to try out new methods. What he learned from Hermann and Flesch stimulated him to experiment and to improve upon their methods. He is known not only for his introduction of many anilin dyes into cytological work and for his fixing fluid, but also for his keen and accurate accounts of cell structure and activity.

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[1]A very similar procedure had previously been employed by Böttcher as explained in a preceding paper of this series (STAIN TECHN., 5, 6, 1930.)

In the late seventies, as each new anilin dye came from the German chemical industries, we find a host of biologists ready to try it out on tissues and the literature of this time is profuse in its praises of this or that dye for each particular purpose. Flemming was especially attracted by the nuclear staining dyes, safranin, dahlia, and magdala red. Eosin became quite popular and was introduced as a counterstain for hematoxylin. It is noteworthy that at this time Paul Ehrlich (1877) was engaged in a study of the action of anilin dyes on cell granules and his work was of tremendous importance to the understanding of the action of stains on cellular structures.

Several Americans, notably Minot, Whitman and Mark studied in Germany about this time and brought back with them an interest in microscopical technic. These men were introduced to the use of the microtome at Leipsig. Improvements in sectioning were being brought about by the advances in paraffin embedding by Anders, Giesbrecht and Mayer at Naples. Microtomes of the sliding type were being perfected, the Naples clamp on the Thoma Jung microtome became well known thruout the biological world. Minot himself later perfected the rotary microtome which permitted the cutting of serial sections as thin as needed for the best cytological and embryological work. The preservation and handling of tissues, proper embedding, and the cutting and mounting of perfect series of sections on slides, all made it possible to introduce more exact and intricate methods of staining.

Not everyone, however, was enthusiastic about the anilin stains. Thus Bolles Lee in his first edition of the Microtomists Vade-Mecum (1885) stated on page 105:

"The great advantages offered by the majority of these dyes are that they stain most animal tissues almost instantaneously and with great brilliancy, whilst the stain readily takes effect on tissues hardened in chromic acid which latter is, as we shall see, in certain cases, a very important point. A decided disadvantage attaching to the whole group is the doubtful permanence of the stains and a further disadvantage of most of them is that the stain is extracted by alcohol or by the clearing media, thus rendering necessary special precautions for the preservation of stained specimens." On p. 107, he adds: "From an examination of the literature it must be concluded that very few of the anilin stains at present known are capable of being useful to the morphologist—to the pathologist they may be of far greater importance."

We also find various investigators disgruntled about their results. Safranin was a bugbear. Many commercial safranins were of no use to the microscopist and gave disappointing results. With a need for anilin dyes particularly suited to the biologist came the establish-

ment of Dr. Grübler's laboratory in Leipzig. He found what the biologist needed and evidently his products gave good satisfaction for they were praised highly and Lee in his subsequent editions of the Vade-Mecum endorsed them enthusiastically. Thus not only the technic of Germany but the dyes themselves came to all parts of the world where staining was done. Perhaps no book has served to disseminate knowledge of microscopical technic more than the Vade-Mecum, which has gone thru numerous editions, in English in French (Lee-Henneguey), and in German (Lee-Mayer) with notable addition by Paul Mayer himself who up to his death was an ardent microscopist in every phase of the work.

In the late eighties and early nineties we find that triple anilin stains were introduced into cytological and histological work. Ehrlich's classification of stains, his theory of staining and his many advances in the uses of new dyes had added much to the knowledge and rational use of anilin dyes. His "triacid mixture" methyl-green, acid fuchsin and orange G, while primarily intended for the differential staining of granules in white blood cells, was adapted by Heidenhain (1888), with the assistance of Biondi, to the use of tissue, especially thin sections, and has served as a very useful cytological stain up to the present time. That such mixtures are difficult to use and capricious in their results must necessarily be true. To duplicate an original formula would be well nigh impossible owing to the variability of the dyes and their actual dye content. Thus the investigator must himself modify his mixture until the proper result is obtained.

Another method which remains from this period is the Flemming (1891) triple stain in which safranin was followed by gentian violet and then decolorized in orange G. This is still a very popular stain for cells in mitosis and a favorite with many botanical cytologists. It is in fact sufficiently important so that a direct translation of the technic as given by the author (1891, p. 296) seems of interest:

For staining I have experimented with a method which if successful brings out the central bodies, the spheres and spindle fibers very sharply: long pre-treatment with weak osmium mixture or with Hermann's solution, washing with water, double staining successively with safranin and gentiana; then the object, after rapid rinsing off of the gentiana, is brought into a concentrated aqueous solution of orange. In this (acid) solution the bulk of the gentiana color is removed little by little; when only very weakly violet clouds form in the dish upon agitation, the object is carried over into absolute neutral alcohol until it loses little or no more color, then into cedar or bergamot oil and finally into damar or Canada balsam.

Of the double stains in use in cytological work, Benda's (1891) light-green-safranin technic gives fine results and lends itself well to photography. This procedure as given by its author is as follows:

Celloidin or paraffin sections are treated 24 hours in:

safranin	1 g.
anilin water	90 g.
alcohol	10 g.

They are then washed half a minute in:

light green FS, or acid violet	0.5 g.,
alcohol	200 g.

They are then passed thru absolute alcohol, bergamot oil, toluol and into Canada balsam.

This technic has the one drawback that light green fades rapidly if the slide is exposed to bright light, a difficulty which can, however, be largely overcome, as shown recently by Haynes (1928), by the substitution of fast green FCF.

Still in general use also is the Van Gieson (1889) stain (picric acid and acid fuchsin), used especially after hematoxylin to decolorize nuclei and stain cytoplasmic elements.

Methylene blue and eosin in various forms are exceedingly useful in cell study and are destined to become more important to the cytologist as our knowledge of the azures and thionins becomes more complete. Various cytologists have used polychrome blood stains, especially Giemsa stain, on thin sections with excellent results. With the work of MacNeal, of Holmes and French and others in the analysis of these polychrome stains, we will be better able to make definite and separate solutions of their ingredients and to control the staining of the cells much more accurately.

As in any other line of investigation, so too in cytology, enthusiasm runs in one direction for a time, and then in another. In the seventies and eighties when the fundamental phenomena of mitosis, fertilization and maturation were being worked out, chromatin dyes were of course all important. Again after the rediscovery of Mendel's law in 1900 we find an increased impetus given to the study of chromosomes in order to link up as much as possible the cytological phenomena with the facts of heredity. Then after many valuable details were discovered attention of cytologists turned to cytoplasmic structures and at present these occupy the center of the field.

Mitochondria under a host of names had been seen in cells by many of the early masters such as Flemming, Strasburger, Altmann and others; but Benda (1901) really first brought them into prominence. Benda's technic of preserving these bodies, and staining with crystal-violet and alizarin, furnishes a method which, with

slight modifications by Meves and Duesberg (1908), has remained extremely useful. To this was added the various methods of Bensley (1911) using especially methyl green and acid fuchsin. For supravital staining of mitochondria he introduced a most excellent dye in Janus green, the popularity of which is in no small extent due to Cowdry.

Aside from mitochondria no other group of cytoplasmic structures seem more important than the so-called cyto-reticular apparatus (called by various names such as Golgi apparatus or canal system of Holmgren, trophospongium and so on), and this is being studied by many cytologists working on plant and animal tissues. To bring out this apparatus as yet the anilin dyes play a very unimportant part. Neutral red has been used and has been claimed by some to be a stain for the Golgi apparatus. This fact is considerably disputed by other cytologists, who believe that the cytoplasmic structures described were vacuoles. As yet, therefore, no specific, well recognized dye for the Golgi apparatus has been discovered, and so we are at present largely limited to the use of rare metals to darken these structures. Gold, silver, osmium, and uranium are used. The methods of Golgi and Cajal with certain recent modifications are employed almost universally.

In conclusion we must not overlook the growing use of the microdissecting apparatus in research on the cell. Here is a new field for the use of stains which in minute quantities can be introduced within the body of the cell and their action observed under high magnification. Here perhaps the vital stains will be most important as an aid to the study of cell physiology. And thus with changes of interest and with the introduction of new mechanical devices we can look forward to an increased demand for new or more refined dyes as important tools of the cytologist.

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HISTORY OF STAINING

THE STAINING OF BLOOD AND PARASITIC PROTOZOA

H. J. Conn, *Agricultural Experiment Station, Geneva, N. Y.*

By the term "blood stain" one ordinarily means a compound dye formed from the chemical union of an acid and a basic dye, and usually a compound of the eosin-methylene-blue group. It is well known today that the sodium salt of a color acid (e. g. eosin) and the chloride of a dye base (e. g. methylene blue) may be converted by simple metathesis into sodium chloride plus the compound dye (e. g. methylene blue eosinate), the latter being insoluble in water unless an excess is present of either the acid or the basic dye. In modern blood stains a compound dye of this type is dissolved in methyl alcohol and mixed with water on the slide at the moment of staining.

If asked who was the originator of such blood stains, the average biologist today would undoubtedly cite Romanovsky (1891) whose classic work on staining the malaria organism with a mixture of eosin and methylene blue has served to attach his name to all stains of this type. As a matter of fact, however, more than a decade earlier Ehrlich (1879) had employed compounds of acid and basic dyes for staining blood cells, and apparently had a better understanding of the chemistry involved than did Romanovsky when he first combined eosin with methylene blue. Ehrlich seems to have been the first biologist to recognize the difference between acid and basic dyes and to appreciate the significance of that difference in staining. He explained how such dyes could be combined by the metathetical reaction above described and called the resulting compound dyes "Neutrale Farbstoffe," a term which we translate *neutral stains* rather than *neutral dyes*, because dye chemists employ the latter English term in a different sense. Ehrlich discussed the insolubility of such compounds in water but stated that they could be dissolved in an excess of the acid dye, and he employed them in a solution thus prepared. For this purpose he mixed five volumes of saturated acid fuchsin, for example, with one volume of strong methylene blue solution, shook, added five volumes of water, and filtered after standing. With this compound dye he apparently obtained a picture similar to that given by modern blood stains, the neutrophile granules appearing violet in distinction from the basophile and acidophile elements.

Romanovsky's important contribution in 1891, therefore, was not the idea of combining an acid with a basic dye for staining blood;

and, altho he is admittedly the first one to have employed a methylene-blue-eosin combination for this purpose, his chance use of these two dyes was a comparatively minor matter after Ehrlich's announcement of the principle twelve years earlier. His outstanding contribution seems rather to have been the demonstration of the nucleus of a parasite in the blood with this compound stain, as the result of which interest in such stains was greatly stimulated. This is undoubtedly the reason why modern blood stains, differing almost as much from his as from Ehrlich's earlier neutral stain combinations, are generally known as modifications of Romanovsky's. Romanovsky, moreover, was probably the first to obtain the blood picture which we regard as typical today and which a satisfactory modern blood stain is expected to give regularly. His securing such a picture, nevertheless, seems to have been largely accidental. He did not intentionally polychrome his methylene blue, and he did not separate out the neutral stain from the mixture of uncombined acid or basic dye; but he did remark that old methylene blue solutions (on which a scum had formed) gave better results than fresh ones. He also realized that some new dye was formed in the preparation of his staining fluid, giving a red color to nuclei. This dye was denoted in the literature for some time merely as "das Rot aus Methylenblau" and the true chemistry of its actual components, the azures, was not understood until very recently. We realize today, therefore, that Romanovsky must have had some of the azure eosinates present in his staining fluid; in its method of preparation, however, it was merely a mixture of aqueous methylene blue of uncertain age with aqueous eosin, used before appreciable precipitation had time to occur. Probably such precipitation was largely prevented by an excess of one dye or the other.

During the same year it happened that Unna (1891) studied the ripening of methylene blue and hematoxylin, publishing the formula of his polychrome methylene blue, as explained in an earlier paper of this series (Stain Techn. **5**,9). This was an alkaline methylene blue prepared by adding sodium, potassium, or ammonium carbonate, which on standing is sure to be converted in part into the azures. Polychrome methylene blue alone was not used for a blood stain; but it is an important constituent of nearly all modern stains used for blood films.

Nocht (1898) contributed the next important step toward the development of modern blood stains in using Unna's polychrome methylene blue in the Romanovsky combination. Nocht concluded the differential staining obtained by Romanovsky was due in part to

decomposition products of methylene blue; and recognizing what Unna had done in the way of intentionally producing these decomposition products, he decided to use the polychrome methylene blue recommended by the latter. First he neutralized the latter solution, using litmus paper to indicate its reaction. He stated that litmus paper could be thus employed by noticing the color in the zone above that stained by the dye itself. Neutralization was accomplished by adding acetic acid until a distint red appearance was given to litmus paper; this was followed by neutralization with alkali until no red color was observed. This neutralized polychromed solution was then mixed with ordinary methylene blue solution until the violet shade due to the polychroming had disappeared; and was then mixed with eosin. He observed a fine precipitate, but said it was not troublesome.

Nocht did not realize the value of that precipitate; but Jenner (1899) at about the same time collected it and found it a useful stain. Jenner probably had not seen Nocht's paper and did not polychrome his methylene blue. Instead he mixed ordinary methylene blue with eosin, as Romanovsky had done; but instead of staining with the mixture, he filtered off the precipitate formed and redissolved it in methyl alcohol. This methanolic solution he employed as a blood stain in much the manner blood stains are used today. His stain lacked the nuclear staining principle of Romanovsky's and Nocht's stains; but it is still occasionally used today. It is hard to see how anyone today can prefer it to other blood stains; but at the time when it was proposed it was an important step, showing that the staining principle of Romanovsky's stain was in the precipitate rather than in the part remaining in solution. A practically identical stain was employed by May and Grünwald (1902), and is in some places called after them rather than after Jenner.

The next step, that of applying Jenner's procedure to the Nocht stain, was such a logical one that it is not surprising to find it occurring to more than one worker simultaneously. Two independent workers, in Germany and England respectively, published papers two years after that of Jenner's in which the procedure was described of collecting and redissolving the Nocht stain precipitate. Reuter (1901) used anilin oil and absolute alcohol as the solvent; Leishmann (1901) dissolved the precipitate in methyl alcohol. Modern blood stains are usually modifications of Leishmann's differing from it only in detail.

In America of recent years the term *Wright's stain* has come to be almost synonymous with blood stain, to such an extent that stain companies say it is hard to sell one unless it is so labelled. Wright's

stain, however, is really a Leishmann stain, differing from the latter only in that the polychrome methylene blue is prepared by heating with sodium bicarbonate for only an hour in flowing steam instead of with sodium carbonate for 12 hours at 65°C, followed by ten days at room temperature. The original popularity of Wright's stain was due to the greater rapidity with which it could be prepared. Now that biologists buy it already prepared by stain companies, however, they care little whether it is prepared by the method of Leishmann, Wright, or in some other way, as long as it gives the proper results. As a matter of fact, the manufacturers usually do make it by a different procedure; but sell it labelled Wright stain in order to meet the demand.

Stains of the Leishmann-Wright type are thus prepared by starting with indefinite mixtures of simple dyes, and the product is naturally a still more indefinite mixture of compound dyes. Frequent attempts have been made, however, to obtain stains giving similar blood pictures but prepared from compounds of definite chemical composition. It had been recognized quite early by a chemist, Bernthsen (1885), that methylene blue readily yielded certain oxidation products which he called methylene azure and methylene violet. This observation was significant from the standpoint of blood stains, altho it was sometime before this significance was appreciated by any biologist. Romanovsky did not realize that his "Rot aus Methylenblau" had been thus described by a chemist several years earlier. Nocht was familiar with methylene violet; but could not identify the "Rot aus Methylenblau," merely stating that it was neither methylene blue, nor methylene violet.

Michaelis, who as we have seen (Stain Techn. 5, 10) made the most important contribution to the chemistry of fat staining, was also the first (1901) to show the relation between Bernthsen's chemical work and the staining results obtained by Romanovsky. He realized that methylene azure was the dye recognized by the latter as "das Rot aus Methylen Blau," and thus contributed distinctly to an understanding of Romanovsky's and Nocht's stains. Bernthsen had been somewhat mistaken, however, in regard to the chemical structure of methylene azure; and Michaelis accepted Bernthsen's chemistry without realizing the error.

Before anyone had corrected this error in regard to the chemical composition of the azure, Giemsa (1902 a & b, 1904) began putting some of Michaelis' principles into practical use. Giemsa obtained reasonably pure methylene azure and methylene violet, combining each of them with eosin and then testing their staining properties.

Methylene violet he regarded as useless; methylene azure gave a good Romanovsky stain except that the blue coloration of the cytoplasm was lacking. By adding a certain amount of methylene blue, the latter could be secured. As a result of Giemsa's work the Grübler Co. put on the market three products: azure I, a fairly pure preparation of Bernthsen's methylene azure; azure II, a mixture of azure I in equal parts with methylene blue; azure II-eosin, an eosinate of azure II, in other words a complete blood stain prepared without the uncertainties of polychrome methylene blue. In his 1904 paper Giemsa recommended 3.0 g. azure II-eosin ground finely with 0.8 g. azure II, sifted thru a fine sieve into 250 g. glycerin and dissolved at a temperature of 60°C; then mixed with 250 g. methyl alcohol at the same temperature; and filtered after 24 hours at room temperature.

A correct understanding of the chemistry of methylene azure was first obtained by Kehrmann (1906) and Bernthsen (1906) who were working in close coöperation. Kehrmann showed the presence of two compounds in azure, trimethyl thionin, and asymmetrical dimethyl thionin, while he obtained symmetrical dimethyl thionin by synthesis. His conclusions were accepted by Bernthsen who admitted that the formula first published by him was incorrect. MacNeal's first paper (1906) on the subject appeared almost simultaneously with that of Kehrmann's. Not having seen the latter, he accepted Bernthsen's original idea as to the chemistry of methylene azure. The chief importance of this paper of MacNeal's was his historical discussion concerning blood stains and the publication of various formulae preliminary to the tetrachrome stain proposed by him later.

MacNeal's tetrachrome stain (1922) was originally published without taking account of Kehrmann's work showing that there was more than one azure. The formula given was: eosin, water soluble, 1 g.; methylene blue, medicinal, 1 g.; methylene azure, recrystallized, 0. 6 g.; methylene violet, recrystallized, 0.2 g.; methyl alcohol, pure (Merck's reagent), 1000 cc. A little later MacNeal (1925) took Kehrmann's work into account, naming trimethyl thionin azure B, and the asymmetrical dimethyl compound azure A. He then specified that azure A should be employed in the tetrachrome stain in the quantity above indicated; and at the same time showed a simpler method of preparing azure A from methylene blue than any that had been proposed previously. The following year Holmes and French (1926) contributed further to methods of preparing these oxidation products of methylene blue, and showed how to produce still another azure, mono-methyl thionin, which they named azure C. This latter dye, primarily of value in tissue staining, is also an ex-

cellent substitute for azure A in the tetrachrome stain, altho the literature seems to contain no reference to its use for the latter purpose.

One important phase of blood staining has always been the demonstration of parasites in the blood. Thus Romanovsky's work was primarily a study of the malarial organism; and a satisfactory demonstration of this organism in blood has always been regarded as important a requisite of a good Romanovsky stain as the proper distinction between the various types of blood cells. Giemsa's earlier work was largely concerned with the staining of the malarial organism and his later papers (1905, 1907, 1909) with that of other parasitic protozoa or protozoal-like organisms, notably that of the syphilitic parasite. The latter, *Treponema pallidum*, has proved such a difficult organism to stain that the literature is full of different procedures for the purpose, none of them regarded as entirely satisfactory. Giemsa's latest procedure is to fix the smear of serum in Schaudinn's sublimate alcohol, to treat with an iodine-potassium-iodide solution, then with sodium thiosulfate, and finally with a freshly diluted Giemsa stain (one drop of the formula given above diluted to 1 cc.). Such a preparation is then run thru acetone into xylol and mounted in cedar oil.

Giemsa's stain shows the syphilis organism red while other spirochaetae are blue. It is probably one of the best of the stains for this organism employing anilin dyes. It should be observed, however, that the later silver impregnation methods such as that of Levaditi (1905, 1906) give splendid preparations both of the organism in tissue and in smears of serum. Such methods depend on treatment with silver nitrate, exposure to light and development either in a pyrogallate solution or in a photographic developer.

Pappenheim (1912) in his "panoptic universal stain" applied the May-Grünwald stain first and then followed this with Giemsa staining. The resulting preparations give a picture not very different from that obtained with the Giemsa method alone.

Staining with the various methylene-blue-eosin combinations is usually applied to blood smears fixed by drying at room temperature. It can sometimes be employed to advantage following fixation in osmium vapor. In this way slightly different colors are obtained, especially in that the red corpuscles appear green instead of reddish.

Staining of unfixed blood (vital staining) is often undertaken largely for the sake of counting platelets and reticulated corpuscles. The latter cannot be distinguished in fixed preparations. Dyes employed in the vital staining are: methylene blue, toluidine blue,

thionin, brilliant cresyl blue, dahlia, the azures, methyl and crystal violets, neutral red, neutral violet, fuchsin, pyronin, safranin, methyl green and Janus green B. Blood cells are such delicate objects that mixture with aqueous solutions of dyes or even dyes in physiological salt solution often causes changes in them. Pappenheim (1895) therefore introduced into the blood small particles of dry neutral red; while Levaditi (1901) dried an alcoholic solution of a stain on a slide and then placed a drop of blood upon it. Robertson, however, (1917) obtained very good preparations by mixing with brilliant cresyl blue in physiological salt solution; this method is now quite widely used for counting reticulated cells and platelets. Buckman and Hallisey (1921) showed that if brilliant cresyl blue is not available, crystal violet may be substituted for it in this same technic.

A recent contribution along a slightly different line may well be of importance in pointing out a possible future line of development for blood stains. Geschickter (1930) has proposed promising methods for applying "neutral stains" to the study of tissue in neoplastic growths. As it was desired to apply these stains to frozen sections of fresh tissue, and since such sections cannot be stained with alcoholic solutions, it was necessary to select a solvent for the compound stain that would permit mixture with water. Geschickter employed ethylene glycol, and succeeded in mixing a modified Giemsa stain dissolved therein with water plus a small percentage of alcohol, without precipitation. This contribution, without any question, renders neutral stains applicable to a greater variety of procedures and may be an important step in the perfection of stains for blood.

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PICRIC ACID AS A DESTAINING AGENT FOR IRON ALUM HEMATOXYLIN

HSU-CHUAN TUAN,[1] *Dept. of Botany, University of Pennsylvania, Philadelphia, Pa.*

ABSTRACT: **A saturated aqueous solution of picric acid is used to differentiate paraffin sections and smeared pollen-mother-cells stained in Heidenhain's and Delafield's hematoxylins. The method proceeds as usual, except that the iron alum in the destaining process is replaced by picric acid. Mixtures of picro-sulfuric acid and dilute Delafield's hematoxylin and mixtures of picric acid and aqueous hematoxylin have also been tested, of which the latter yields the better result, but is not as good as the other method described here.**

In connection with the author's research work on Clarkias in 1927–28, an unusual destaining agent was tried out for iron alum hematoxylin. A saturated aqueous solution of picric acid was used to differentiate sections previously stained by Heidenhain's method. Picric acid has previously been used to differentiate hematoxylin stained material,[2] but the method has received very little attention, and its applicability to modern cytological researches has not been sufficiently studied. Preparations thus made have proved very satisfactory, and enable the author to count the chromosomes of *C. elegans* without much difficulty, which had not been practicable after differentiation with iron alum. The advantage arises from the fact that the picric acid primarily destains the cytoplasm and leaves the chromosomes practically unchanged. After mounting, the chromosomal image is well defined in a clear cytoplasm which is a result obtained only with great care and precision by using iron alum as the destaining agent, as this chemical has a great tendency to stay in the cytoplasm and gives a very objectionable coloration to the preparation as a whole. Later the same method was applied to many other plants to test its value. They all show good results; and the method is therefore offered here in hopes of its being given wider application.

The method is very simple. Slides which have been mordanted in iron alum and stained in hematoxylin are transferred, two at a

[1]Fellow of the Chinese Educational Mission at Washington, D. C. of Tsing Hua University, Peiping, China.

[2]Zimmerman, A. Botanical Microtechnique. *Transl. by* J. E. Humphrey. Henry Holt & Co., New York, 1893. See p. 182.

time, into a Petri dish with enough aqueous picric acid solution to cover the sections. The slides should then be examined from time to time under the microscope until the correct stage of differentiation is reached. While waiting, two slides may be put in another dish and the number of minutes required for each process recorded, so that when a second batch of slides is ready they may be handled in a wholesale manner without giving them special attention. The following schedule will indicate how the method was applied to the writer's material.

A. For paraffin sections of *Clarkia concinna* ("red ribbons") buds.

1. Mordant in 4% iron ammonia alum for 60 minutes.
2. Wash in running water for 10 minutes.
3. Stain in 0.5% hematoxylin for 10 minutes.
4. Wash in running water for 10 minutes.
5. Destain in a saturated aqueous solution of picric acid.
   a. 10 minutes for quartets.
   b. 30 minutes for spireme.
   c. 90 minutes for dividing chromosomes.
6. Wash in running water for 20 minutes.

B. For paraffin sections of *C. elegans* root tip.

1. Mordant in 4% iron alum for 30 minutes.
2. Wash in running water for 10 minutes.
3. Stain in 0.5% hematoxylin for 30 minutes.
4. Wash in running water for 10 minutes.
5. Destain in a saturated aqueous solution of picric acid for 2½ hours for metaphase plate stages.
6. Wash in running water for 20 minutes.

Delafield's hematoxylin was also employed in this experiment; it gives a closely similar result with picric acid as the destaining agent. For root tips of *Vicia faba* (broad bean), *Sequoia sempervirens* (redwood), and stem tips of *Araucaria bidwilii* (bunya-bunya), Delafield's takes less time in the destaining process and is, perhaps, easier to handle:

1. Stain in undiluted Delafield's hematoxylin for 30 minutes.[3]
2. Destain in a saturated aqueous solution of picric acid until the nucleus shows a brownish color.
3. Wash in water until the nucleus is of a rich blue color.

Later, in 1929, the author was given an opportunity to apply this method on pollen-mother-cell smears at the University of Pennsylvania. Results obtained, tho varying in minor details, were, as a

[3]The stock used was from the laboratory of Prof. B. B. Burlingame of Stanford University; the author has not compared the result with stains from other sources.

whole, quite consistent. *Gasteria* smears will serve as an example to illustrate the general application:

1. Fix smears in Taylor's[4] modification of Flemming's solution for 20 minutes.
2. Wash in running water for 20 minutes.
3. Bleach in aqueous hydrogen peroxide solution (diluted to 33% strength) for 20 minutes.
4. Wash in water for 20 minutes.
5. Stain in 0.5% hematoxylin[5] for 20 minutes.
6. Wash in water for 10 minutes.
7. Destain in a saturated aqueous solution of picric acid.
   a. 20 minutes for pollen grains.
   b. 40 minutes for quarters.
   c. 100 to 120 minutes for dividing chromosomes.
8. Wash in water for 20 minutes.

It is by no means necessary that the above mentioned schedule should be followed with absolute exactness; care must be taken, however, during the final washing in order to remove all the picric acid from the sections and to assure proper differentiation. Sometimes the final washing can be abbreviated by using a diluted solution of ammonia-water (2 drops of ammonia-water in 100 cc. of water), but for a critical preparation, running water is more desirable. Thus in a perfectly destained slide, the cytoplasm should not show a bluish color; if it does the sections can be brought into the picric acid again for another 5 to 15 minutes. The following trial schedule will help the writer to explain the situation better:

1. Mordant in 2% iron alum for 20 minutes (preferably fresh solution).
2. Wash in water for 10 minutes.
3. Stain in 0.5% hematoxylin for 20 minutes (preferably fresh solution).
4. Wash in running water for 10 minutes.
5. Destain in a saturated aqueous solution of picric acid for 60 minutes.
6. Wash in a diluted solution of ammonia-water for 1 minute.
7. Examine under microscope; if the cytoplasm shows a blue stain, the differentiation is not complete, then destain in the same picric acid solution again for 5 to 15 minutes. Repeat 6 and 7 until the desired differentiation is reached.

[4]McClung, C. E. Handbook of Microscopical Technique. Paul B. Hoeber Inc., New York. 1929. See p. 116–119.

[5]The hematoxylin employed in this later work was of American manufacture. Certification No. F H 4 b.

In writing up this article, the author was reminded of the Conklin's[6] modification of Delafield's hematoxylin method for animal eggs. The stain employed in this method is a mixture of picric-sulfuric acid and diluted Delafield's hematoxylin. Due to the lack of material and time, the author did not try it out intensively to judge its applicability to plant material; but a few words about the picro-hematoxylin mixtures tried by the author are not out of place. Mixtures of aqueous hematoxylin and picric acid in different proportions were used on *Gasteria* smears. It seems that there is a little advantage in these mixtures in so far as the time element is concerned, and slides stained in them often show after dehydration a purplish or muddy cytoplasm, which cannot be removed by repeated destaining as it could be done in the foregoing cases. The best result the author has so far obtained with a picro-hematoxylin mixture was by using the following schedule:

1. Mordant in 2% iron alum for 5 minutes.
2. Wash in water for 10 minutes.
3. Stain in a mixture of picro-hematoxylin for 2 minutes (25 cc. of 0.5% aqueous hematoxylin to 25 cc. of saturated aqueous solution of picric acid).
4. Wash in water for 10 minutes.
5. Destain in a saturated aqueous solution of picric acid for 60 minutes.

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[6]McClung, C. E. Handbook of Microscopical Technique. p. 469.

BLOCK STAINING OF NERVOUS TISSUE WITH SILVER

Studies of Fixatives, Lipid Solvents, and Reducing Solutions.

H. A. DAVENPORT, *Institute of Neurology, Northwestern University Medical School, Chicago.*

ABSTRACT—**Silver stains of the Cajal type, made on spinal cord of cats were studied to determine the limits of favorable ammonia concentration in alcohol as a fixative and the comparison of ammoniated alcohol with alcohol-chloroform and alcohol-pyridine mixtures. Such material subsequently extracted with various lipid solvents showed staining of a generally similar character. More intense staining was seen after the alkaline fixatives and best penetration of stain into the blocks after the most thoro extraction of lipids.**

Experiments with reducing solutions which contained various proportions of pyrogallic acid and formalin indicated that pyrogallic acid is the essential ingredient.

Post-mortem autolysis up to 5 hours caused no change in fiber staining.

Many formulae for staining nervous tissue have been developed by Cajal[1] and leave little to be desired in empirical procedures. In 1910 he systematized the various fixatives commonly used in neurological technic according to the effect which they had on subsequent staining with reduced silver. Both aqueous and alcoholic solutions were used, and in addition results obtained by fixation in pure pyridine were described. He found that while fixation in an aqueous medium or in 50% alcohol sufficed for the demonstration of nonmedullated nerve fibers and cell bodies, fixation in alcohol or extraction of the tissue with absolute alcohol before immersion in the silver bath was desirable for the impregnation of medullated fibers. An alkaline fixative (ammoniated alcohol) caused cell nuclei to stain lightly and an acid fixative (formol-acetic acid) caused them to stain vigorously. Fixation in a mixture of equal parts of pyridine and water followed by pure pyridine was recommended for em-

[1]Cajal, S. Ramon, Algunos métodos de coloración de los cilindros ejes, neurofibrillas y nidos nerviosos. Trab. Lab. Biol., Univ. de Madrid, **3**, 1. 1904.

Las formulas del proceder del nitrato de plata reducido (y sus efectos sobre los factores integrantes de las neuronas). Trab. Lab. Biol., Univ. de Madrid, **8**, 1. 1910.

Un procédé simple pour imprégner les gros et les fins axons dans les coups de pièces indurées en formol et quelque autres formules utiles pour des cas particulier. Trab. Lab. Biol., Univ. de Madrid, **26**, 1. 1929.

bryonic material. The flexibility of the methods has been shown by numerous modifications which were evolved in other laboratories.

One of the variants consisted in the use of Carnoy's fluid for the fixative as applied in the methods of Cowdry[2] and of Campbell,[3] while another consisted of pyridine extraction following fixation in ammoniated alcohol (Ranson).[4]

The use of pyridine after alcohol fixation for the purpose of insuring complete extraction of the lipids from tissue of the central nervous system has been criticized by De Castro[5] who refers to its use as a "double fixation." The use of pyridine after formalin fixation as employed in the Bielschowsky method for blocks seems to have a logical theory of application in that the pyridine dissolves the lipids and leaves the nerve fibers readily permeable by aqueous silver nitrate.

In order to consider the function of reagents used in preparing tissue for silvering, one should differentiate between fixatives (protein precipitants) and lipid solvents. Fresh tissue of adult mammalian central nervous system can be regarded from a physical standpoint as a two-phase system—a water phase and a lipid or fat phase. The former comprises the cell bodies and processes while the latter includes the myelin sheaths of the white and other fatty material of the gray matter.

If the fixative (such as absolute alcohol, alcohol-chloroform mixtures, or pyridine) is in itself a lipid solvent, subsequent extraction should not be necessary provided the fixative is allowed to act long enough.

The present work was undertaken to study the reactions of tissue of mammalian central nervous system toward the Cajal type[6] of silver staining. It had for its object a better understanding of the mechanism of silver staining rather than the development of any particular technic or modification of methods.

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[2]Cowdry, E. V., The relation of mitochondria and other cytoplasmic constituents in spinal ganglion cells of the pigeon. Int. Monat. Anat. u. Physiol., **29**, 473. 1913.

[3]Quoted from Schneider, A. J., The histology of the radix mesencephalica N. trigemini in the dog. Anat. Rec., **38**, 321. 1928.

[4]Ranson, S. W., Non-medullated nerve fibers in the spinal nerves. Am. J. Anat., **12**, 67. 1911.

[5]De Castro, F., Technique pour la coloration du système nerveux quand il est pourvu de ses ètuis osseux. Trav. du Lab. Recherch. Biol. de l'Univ. Madrid, **23**, 427. 1926.

[6]This type is characterized by reduction of the silver nitrate directly without the use of an ammoniated silver solution as used in the Bielschowsky procedure.

Experimental

Spinal cords of adult cats were used for material. These were usually put into the fixative from 1 to 1½ hours after death of the animal, but when the post-mortem time was varied, the samples were given identical treatment in staining so that an observation of the effect of post-mortem autolysis could be made. Likewise when the technic of staining was varied the samples were all fixed at the same time.

Some preliminary trials had indicated that the use of heavy metal salts (such as mercuric chloride, chromium fluoride, and uranyl nitrate) in aqueous solutions as fixatives resulted in poor penetration and uneven fiber staining, especially in the gray matter. Hence the

Table 1. Schedule of Variations in Fixation and Subsequent Extraction of Eight Blocks of Cat Spinal Cord.

Specimen No.	40	41	41P	42	42P	43	43P	44
Fixed 3 days in	Abs. alc. 2 pts. $CHCl_3$ 1 pt.	Abs. alc.		Abs. alc. + 1% ammon. water		Abs. alc. + 5% ammon. water		Abs. alc. + 20% ammon. water
4th day	$CHCl_3$ to W	to W	*Py	to W	Py	to W	Py	to W
5th day	2½% $AgNO_3$ (aq)	†Ww		$AgNO_3$	Ww	$AgNO_3$	Ww	$AgNO_3$

All specimens silvered at 39°C for 6 days. Reduced 1 day in pyrogallic acid, 3%, formalin, 3%.

*Py = Pyridine (Merck's medicinal).

†Ww = Changes of distilled water.

fixatives have been limited almost entirely to lipid solvents in subsequent experiments. Such fixatives fall into the neutral class according to Tolstoouhov's[7] classification, in that they do not greatly alter the isoelectric point of natural protein during fixation.

Table 1 gives the plan of treatment of eight blocks (5 to 7 mm. in length) of cat spinal cord for comparing the effect of ammoniated alcohol with absolute alcohol and alcohol-chloroform mixture as fixatives. "P" specimens were given a supplementary extraction with pyridine.

The tissue was embedded in paraffin. The blocks were sectioned approximately to the middle and sections from varying depths mounted on the same slide for study.

[7]Tolstoouhov, A. V., The effect of preliminary treatment (fixing fluids) on staining properties of the tissues. Stain Techn., 3, 49. 1928.

Subsequent microscopic examination of the specimens showed a similarity of the character of the stain in all except the block fixed in 20% ammoniated alcohol. In this the fibers were pale except in a region adjacent to the central canal and in the extreme periphery of the block. The ganglion cells appeared swollen and were pale and without intra-cellular detail. In the other blocks the stain varied from moderately pale in the chloroform-alcohol fixed specimen to dark in the one fixed in 5% ammoniated alcohol. Nuclear detail was most marked in the plain alcohol fixed specimen. Neurofibrils stained in some of the ganglion cells in all and terminal loops on the cell surfaces were fairly abundant. Neuroglia cells and processes were stained pale yellow and mesodermal connective tissue light brown. The degree of penetration was fair but the centers of the blocks were less well impregnated than that part about 0.5 mm. from the surface.

TABLE 2. PLAN OF TREATMENT OF TWELVE BLOCKS OF CAT SPINAL CORD FOR COMPARING RESULTS AFTER DIFFERENT LIPID SOLVENTS.

Specimen No.	6A, 6B, 6C & 6D	7A, 7B, 7C & 7D	8A, 8B, 8C & 8D
Fixed 2 days in	Abs. alcohol	Abs. alc. + 2% ammon. water	Acetone
Subsequent treatment with a lipid solvent (1 day)	A Pyridine B Chloroform C Abs. Alc. D Xylene	A Pyridine B Chloroform C Abs. Alc. D Xylene	A Pyridine B Chloroform C Abs. Alc. D Xylene
To water thru:	Abs., 95%, 80%, and 50% alc.	Abs., 95%, 80%, and 50% alc.	100%, 80% and 40% acetone

All specimens washed 20 hours with several changes of distilled water, silvered in 1.7% $AgNO_3$ (aq.) for 4 days at 38°C. Reduced 1 day in pyrogallic acid, 3% + formalin, 3%.

Comparisons between the specimens fixed with the same fixative but with and without pyridine extraction showed no differences attributable to the pyridine treatment.

It should be noted that all of the above specimens were washed in water for approximately one day so there would be only traces of pyridine or ammonia carried by the tissue into the silver bath.

Experiment 2 was designed to test the effects of different lipid solvents. It consisted of twelve specimens of cat cord. Three fixatives, absolute alcohol, 2% ammoniated absolute alcohol, and acetone were used. Four blocks of cord (5 mm. thick) were fixed in each. One block from each of these three groups (after 2 days' fixation) was subsequently extracted with pyridine, a second with

chloroform, a third with absolute alcohol, and a fourth with xylene thru acetone. The first three (A, B, and C series, Table 2) were brought thru graded alcohol and the fourth (D series) thru graded acetone to water and washed as in the preceding experiments. Table 2 gives the plan of preparation of this series.

Microscopic study of the preparations indicated that among the three fixatives ammoniated alcohol gave least shrinkage of ganglion cells while acetone gave the most. When considered from the standpoint of the effect of the different lipid solvents, the preparations treated with alcohol alone or alcohol followed by pyridine stained somewhat darker than those treated with chloroform or xylene. Cell nuclei were stained darkest after plain alcohol fixation followed by pyridine and lightest after acetone fixation followed by chloroform. The similarity of the 12 specimens was more in evidence than differences. The three treatments of the three specimens which showed most differentiation between neural elements and neuroglia were: ammoniated alcohol-pyridine, alcohol only, and acetone-chloroform. This is cited to show that no specific inhibition of the staining of glia by any one of the fixatives or subsequent treatments appeared in the series.

In other experiments, not to be given in detail, the following observations were made. Acetone fixation not followed by a good lipid solvent gave poor penetration due supposedly to its being an inefficient myelin solvent. (For schema of solubilities of various lipid constituents of central nervous system, see Maclean[8] or Weil.[9]) Chloroform extracted specimens, after all fixatives used, often exhibited good neurofibrils and lightly stained cell nuclei. Alcohol alone was sufficient to fix and to extract the tissue, and ammonia water could be added up to 5% without much change in the stained product, provided the excess of ammonia was removed by washing in several changes of alcohol or water. Cell nuclei were prominent in tissue fixed in plain alcohol but usually pale after ammoniated alcohol, and neurofibrils were scarce in the former and abundant in the latter. This is in agreement with Cajal's finding in 1910 (see footnote 1) that neurofibrils are best demonstrated by alkaline, and cell nuclei by acid fixatives.

The degree to which pyridine, ammonia or other alkali is removed is probably responsible for some of the variations seen in the intensity of staining. That it is not necessary to wash all of the alkali out of the

[8]MacLean, H. and MacLean, I. S., Lecithin and allied substances. London, 1927.

[9]Weil, A., The influence of formalin fixation on the lipoids of the central nervous system. J. Biol. Chem., **83**, 601. 1929.

fixed tissue is indicated by Cajal's practice of adding pyridine to the silver bath and also the transferring of tissue directly from ammoniated alcohol to silver (Cajal and Campbell methods). In our experiments, less thoroly washed blocks stained more intensely than those washed for 24 hours in many changes of distilled water.

One block fixed 4 days in pure pyridine, washed 6 hours in several changes of distilled water, 2 hours in 50% alcohol, and 6 days in 2% silver nitrate, showed more end bulbs about the ganglion cells than any of the other preparations. Another block fixed in a mixture of equal parts of pyridine and water for 2 days, then put into pure pyridine for 2 days, but otherwise treated the same as the first, showed end bulbs almost as numerous. A third treated like the second except that after pyridine it was transferred to absolute alcohol for 5 hours, then 95% 2 hours, 80% 2 hours, and into the silver bath, showed well stained intracellular fibrils but few end bulbs. These are cited to serve as a guide to the use of water or alcohol for washing blocks after pyridine.

Chloroform extraction must be followed by alcohol because chloroform is not miscible in water, and altho pyridine is miscible in water in all proportions, it would seem better practice to let it be followed by alcohol in order to obviate the possibility of reprecipitating lipids inside of the tissue and also lessening osmotic changes. A comparison of preparations which had been washed in water and transferred from water to the silver bath with others which had been transferred from 80% alcohol (after 1 change each of absolute and 95%) showed, in addition to the differences noted above, somewhat darker staining of ganglion cell cytoplasm, blood cells, and perivascular connective tissue in the alcohol washed specimens.

Since the above experiments indicated that among the essentials involved in fixation for pyridine-silver or Cajal fiber stain were the extraction of lipids and alkalinization of the tissue, a mixture of equal parts of pyridine and 95% alcohol—similar to Cajal's formula D (see footnote 1, 1929 citation) but containing a higher percentage of pyridine—was tried as a fixative. The object of using a fixative which had a high pyridine content was to determine whether there was any specific effect of pyridine other than alkalinization and extraction of the tissue. Blocks were fixed for 2 days and then put into absolute alcohol for 3 or 4 hours, 95% alcohol for a similar period, 80% alcohol overnight and then transferred to the silver bath. The volume of alcohol for each change was 25 to 50 times that of the block to insure removal of the excess of pyridine. Preparations made after this method of fixation were so similar to those

fixed in ammoniated alcohol which contained 2% ammonia water that it is doubtful if there is any real choice between the two fixatives. The pyridine-alcohol mixture was used for fixation of the blocks used in Experiment 3.

Experiment 3 was made to test the efficacy of reducing solutions of different strengths of formalin and pyrogallic acid. All preparations were fixed and silvered according to the schedule given in Table 3.

TABLE 3. LIST OF FORMULAE OF REDUCING SOLUTIONS USED FOR COMPARING THE EFFECTS OF FORMALIN-PYROGALLIC ACID MIXTURES OF WIDELY VARYING COMPOSITION.

Specimen No.		1	2	3	4	5	6	7	8	9	10	11
Fixed 2 days in		Pyridine-alcohol, equal parts.										
Subsequent treatment		Two changes 95% alc.—3 hrs. each; one change 80% alc.—18 hrs., 2% $AgNO_3$—4 days. Rinsed in dist. water 15 sec.										
Reducing solution (aqueous)	Pyro. %	10	10	8	5	2.5	3	1	0.5	0.2	0.1	0
	Form. %	0	4	20	50	25	3	10	50	100	20	20

Subsequent examination of the preparations showed that the pyrogallic acid was the active ingredient, for the first six (reduced in a solution of 2.5 to 10% pyrogallic acid) were well stained, while of the remainder, only No. 7 was usable at all. No. 1, reduced in 10% pyrogallic acid without formalin was one of the best. There was some tendency for overstaining in the periphery of blocks which had been reduced in high concentrations of pyrogallic acid. This experiment indicated that the composition of the reducing solution was uncritical but that in general a high pyrogallic acid content was desirable and since No. 4 was the poorest of the better group, a high formalin content was undesirable. Altho formalin alone (as used in Bielschowsky's method) reduces ammoniated silver, it reduces silver nitrate scarcely at all in an acid medium (pH 5±) at room temperature. In reducing solutions which had been kept over a period of several weeks, the presence of formalin slowed the oxidation of pyrogallic acid by air. However, experiments showed that air oxidation could be prevented almost entirely by adding 0.5% of sodium bisulfite ($NaHSO_3$) to the pyrogallic acid solution.

Observations on the effect of post-mortem autolysis on silver staining have been limited to 12 preparations of which two were taken 20 hours after death, four after 5 to 6 hours and the rest between 1 and 3 hours. The stainability of the nerve fibers showed no diminution during 5 hours, but many fine fibers were poorly stained in the 20 hour specimens. There was usually somewhat better

differentiation between neural elements and neuroglia in tissue taken 1 to 2 hours post-mortem than in the very fresh tissue. This is in agreement with the generally accepted dictum that neuroglia stain best when tissue is fixed as soon as possible. Ganglion cells showed objectionable shrinkage in the specimens fixed 5 hours post-mortem or later.

In an experiment designed to test the time of fixation in 2% ammoniated alcohol, and in alcohol-pyridine (equal parts), one block was fixed in each for 1, 2, 6, and 40 days. The blocks fixed 1 day were not well stained in the centers. This suggests incomplete extraction of lipids. There was little difference between the 2 and 6 day specimens: both were normally stained. The 40 day specimens showed very dark staining of ganglion cells and large fibers but the smallest fibers in the gray matter were pale and pericellular plexuses were unstained to incompletely stained.

In a rather limited series on reduction time, our results indicated that an equilibrium between tissue and reducing fluid is attained within 24 hours.

Discussion

These experiments indicate that the making of fiber stains of the Cajal type of adult mammalian central nervous tissue is a relatively uncritical process provided a good extraction of lipids is effected and, if neurofibrils and fine fibers are desired, the tissue is alkalinized during fixation with pyridine, dilute ammonia, or, as Okada[10] has recommended, a dilute alcoholic solution of sodium hydroxide. This alkalinizing effect appears to be partially neutralized by prolonged washing in distilled water after fixation.

The more thoro washing in distilled water and transfer of blocks from water to the silver bath resulted in greater differentiation between large and small nerve fibers than less complete washing in alcohol and transfer from alcohol to the silver bath. This phenomenon of large fibers stained pale yellow or brown with small fibers black appeared as a matter of intensity of staining governed by fiber size and only coincidentally related to myelination, for it occurred after complete removal of the myelin sheaths with all types of lipid solvents. It is not the same as the phenomenon of selective penetration seen in staining of blocks from which the myelin has not been removed.

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[10]Okada, S., Über eine neue Silberimprägnationsmethode zur Darstellung der Neurofibrillen. Folia Anat. Jap., 7, 403. 1929.

The depth of penetration appeared to be regulated particularly by the concentration of pyrogallic acid in the reducing solution. If superficial structures are desired, one can use a dilute reducing solution but if the deeper ones are desired, pyrogallic acid solutions of 5 to 10% may be used.

No study was made of the effect of varying the time the tissue was left in the silver bath because it seemed to have been demonstrated by Cajal that this is uncritical between 3 to 7 days. Croll[11] impregnated pituitary tissue for various lengths of time and found 7 days at 35°C gave the best results. In our present series, 4 or 5 days seemed preferable to 3. Longer time for impregnation has been used by Shiro[12] for neurofibrils in tissue culture (14 to 28 days)—and by Agduhr[13] for human fetuses (6 to 12 weeks).

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[11]Croll, Margaret M., Nerve fibers in the pituitary of a rabbit. J. Physiol., 66, 316. 1928.

[12]Shiro, E., A sure method for the elective staining of neurofibrillae in tissues cultivated in vitro. Zts. Wis. Mikr., 46, 369. 1929.

[13]Agduhr, E., Einige Beiträge zur Ag-Technik der Stückimprägnierung von Nerven. Anat. Anz., 69, 363. 1930.

CUTTING MICROSCOPIC SECTIONS OF WOOD WITHOUT PREVIOUS TREATMENT IN HYDROFLUORIC ACID

IVAN H. CROWELL, *Dept. of Botany, Miami University, Oxford, O.*

Introductory Note.[1] A technic has recently been proposed by Kisser[2] for sectioning hard materials. Specimens too resistant to section freehand in the ordinary manner are softened by allowing a regulated jet of steam from water heated in a flask to flow over the wood. They then cut readily. Material which is less resistant may be cut by allowing hot water to drop constantly upon it. Kisser has figured a piece of apparatus built under his direction by Reichert, which is useful in the steam method.

It is an interesting coincidence that the same manner of cutting resistant woods was discovered separately by Mr. Ivan C. Crowell of this laboratory quite independent of Kisser's work. A knowledge of this revolutionary method of cutting even the most resistant specimens, including locust woods and vegetable ivory, led us to write to Dr. Charles J. Chamberlain at the University of Chicago to ask whether or not he had knowledge of such a method ever having been used. He replied that he had not until a few days before, when the paper cited above had come to his desk for review. This he sent to us, suggesting that we review it and publish the method of Mr. Crowell in conjunction therewith. The following paper, written by Mr. Crowell, gives the procedure as developed by him and used in this laboratory.—A.T.E.

In microchemical analysis of woods Harlow[3] has shown that woods previously treated with hydrofluoric acid no longer give the same chemical reaction as woods not so treated. The same author has shown that biological stains are unreliable indicators of the chemical composition of wood after such treatment. Thus, it becomes necessary to devise a means of cutting microscopic sections without acid treatment if such woods are to be studied.

The method as used by the author was to boil water slowly in an Erlenmeyer flask from which the vapor was led to the microtome thru a small copper tube in which a few coils had been turned. Another Bunsen burner was placed below the coils to vaporize all the

[1]By Arthur T. Evans, Prof. of Botany, Miami, Univ.

[2]Kisser, Josef. Der Heutige Stand Botanisch-Mikrotechnischer Schneidemethoden. Biologia Generalis, 4, Lieferung 1/2, 151–3. Emil Haim and Company, Wien and Leipzig. 1928.

[3]Harlow, Wm. M. Contributions to the Chemistry of the Plant Cell Wall. Tech. Pub. 26. N. Y. State Coll. of For. Syracuse, N. Y.

water before it left the tube. As the steam slowly flowed over the piece of wood to be sectioned, there was no water of condensation leaving the tube; however, it did condense on the wood, which was kept continually hot and wet. Under these conditions, as many as five hundred microscopic sections of sound beech and a number of sections of vegetable ivory have been cut with one sharpening of the razor. These sections could be studied microscopically and microchemically with resulting true chemical reactions, for its various constituents. Since steam at atmospheric pressure reaches only 100°C, it was assumed that no changes occurred in the woody tissue.

There are other advantages in this procedure: sections may be cut off at once from any wood, are free from air and curling, and may be cut as thin as 10μ. One disadvantage, however, is in using copper tubing. This forms copper oxide on the inside of the coil and is blown out at intervals in the form of a fine black powder, which both dulls the razor and causes a discoloration and foreign particles in the section. This may be overcome by using aluminum or monel metal tubing.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

BAILEY, H. D. A practical flagella and capsule stain for bacteria. Science 72, 95–6. 1930.

Make a thin smear from a 15 to 24 hour agar growth, onto a clean slide, preferably transferred first to a drop of water and allowed to stand therein 5 minutes. Air-dry, without heat.

Cover with mordant (5% tannic acid, 3 parts; 10% ferric chloride 1 part) 2 minutes. This solution will keep indefinitely.

Pour off mordant and cover with following mixture freshly prepared: 7 drops mordant mixed with 1 drop Ziehl-Neelson carbol fuchsin; 1 drop conc. HCl added and mixed; then 1 drop conc. formaldehyde. Apply 7 minutes. Wash in running water.

Cover with Ziehl-Neelson carbol fuchsin (basic fuchsin 10 g.; 95% ethyl alcohol 100 cc. 5% aqu. phenol, 1000 cc.) and steam gently ½ minute. Wash in running water. Blot.

In staining peritoneal exudate to demonstrate capsules of pheumococci, employ following technic:

Spread loopful on glass slide either undiluted or mixed with loopful of water.

Apply mordant for 10 seconds. Wash in running water.

Apply cold diluted carbol fuchsin stain 10 seconds. Wash with water; and blot.

BEERS, C. D. The preparation of permanent slides of the rhizopod Arcella. Science, 77, 122. 1930.

This method supplements Hegner's fixation and preservation in glycerin.

Transfer Arcellae from the stock culture to slides with few drops of the fluid. Leave undisturbed in a moist chamber for ½ hour, during which the arcellae will settle to the bottom and attach themselves to the slides by means of pseudopodia.

Pour off surplus fluid so that only a thin film will cover the animals. Drop Schaudinn's solution (cold) directly on the animals and leave for 2 to 3 minutes.

Transfer to 70% alcohol (made light brown with addition of tincture of iodine) for 30 minutes; 50% alcohol, 25% alcohol, and water, 1 to 3 minutes in each.

Stain with Delafield's hematoxylin (1 part stock solution to 3 parts water) 5 to 10 minutes, or with Heidenhain's iron hematoxylin (mordant 1 hour in 4% iron alum and stain at least 4 hours in ½% hematoxylin,) destain, clear and mount.

This method leaves the animals attached to the slides with pseudopodia extended. Nuclei with their conspicuous karyosomes, as well as the shell aperture and pseudopodia, are splendidly revealed. Chromidia (often overlooked with other methods) are excellently demonstrated. The preparations are clear and permanent.

FOLEY, J. O. Studies in stain technique. II. Cytological staining with Auerbach's methyl green-acid fuchsin mixture. Anat. Rec., 45, 339–344. 1930.

The success of Auerbach's stain in cytologic work and especially for germ cells depends largely upon the fixative used. The following method of mordanting and staining proved satisfactory for testes of *Romalea microptera*, *Passer domesticus* and *Didelphys virginiana*. Gilson's solution, Allen's B 15, and Flemming's strong fluid in Hance's modification were used as fixatives.

Run down paraffin sections to water and place in 1% potassium permanganate for 5 minutes, treat with 1% oxalic acid to remove permanganate. Treat with iodine before mordanting if fixative contains mercury.

Wash in running water 30 minutes, and mordant in 0.5% osmic acid for 12 to 24 hours if none were present in the fixative.

Wash in running and distilled water and stain 24 hours in a mixture of 80 cc. of 0.2% aqu. methyl green (58% dye content) and 20 cc. of 0.1% aqu. acid fuchsin (65% dye content). Both dyes were National Aniline products.

Wipe off excess stain; blot, but do not dry completely.

Transfer to acidulated 95% alcohol for 5 to 30 seconds or more, transfer to carbol-xylol; then xylol, and mount in balsam.

Condensed chromatic structure, stain brilliant green. More loosely arranged chromatin varies from dark blue green to purple. Plasmosomes, spindle fibers and tails of spermatozoa are light pink to brick red. Fibrous connective tissue and muscle stain a very dark red. This stain is especially differential for chromatin nucleoli and developing spermatozoa.

Guilliermont, A. Culture d'un Saprolegnia en milieux nutritifs additionnés de colorants vitaux. Valeur de la méthode des colorations vitals. Bull. d'Histol. Appl., 7, 97–110. 1930.

Saprolegnia (species?), was grown on soy bean agar with 0.001 to 0.005% neutral red at 25°C. Normal growth occurred with 0.001% dye. With 0.002% dye fewer sporangia developed and with 0.005% spores were absent, altho the mycelium was normal up to 0.04% dye. The vacuome may thus be studied from the germination of zoospores till the formation of zoosporangia, since it is stained in all these stages.

Analogous experiments were performed with other vital stains (source not mentioned) for the vacuome and the chondriome on 1% peptone broth and on soy bean agar. After neutral red, toluidin blue is least toxic of the stains tested. Development of the fungus succeeds with 0.001 to 0.03% of the latter dye, and is checked by 0.04 to 0.05%. The mycelium and sporangia fail to stain, regardless of the amount of dye used. Growth is very slow with cresyl blue, methyl blue or nile blue, and no staining takes place. All of these four dyes stain under the microscope, the fungus apparently reducing them in the medium.

All chrondiosome stains proved very toxic. Growth occurs only with 0.003% or less of Janus green, and this starts only after complete reduction of the dye. Development is prevented by as little as 0.001% methyl violet or dahlia. The vacuome dyes, on the contrary, are very slightly toxic, especially neutral red, which is strictly limited to the vacuome and does not alter the normal activities of the cytoplasm.

Hubin, Marguerite. Un procédé rapide de triple coloration. Extrait des Arch. de Biol., 37, 23–29. 1928.

The stain proposed is permanent in solution as well as in sections.

Fix for a considerable length of time in Holland's solution, using 5% trichlor-acetic acid (about 12 drops to 50 cc. of the fluid) instead of the glacial; transfer to Bouin's for 24 hours, then to 95% alcohol and embed as usual.

Wash sections to remove remaining picric acid.

Stain in acid hematein according to Carazzi, ripened a few months (formula may be found in Langeron's manual), 10 minutes for embryonic tissue, 20 to 30 minutes for adult.

Tap water until blue (about 15 minutes).

Differentiate in dilute acetic acid (12 drops glacial to 100 cc. water) for 4 to 5 seconds, and gradually bring up to 70% alcohol.

Stain with agitation for 1 to 3 minutes in the following solution, ripened 8 to 10 months: 1% aqueous eosin A. G. (Grübler), 10 cc.; 1% aqueous gold orange (Grübler), 20 cc.; 1% safranin (Merck) in 50% alcohol, 20 cc.; absolute alcohol 50 cc.

Differentiate for a few seconds in acid alcohol (95% alcohol, 100 cc.; HCl, 2 to 3 drops).

Transfer to: absolute alcohol; xylol; balsam.

Results offer great clarity and differential staining. For example sections of mice embryos show: brilliant yellow blood vessels, violet cartilage, blue zones of ossification, brownish yellow muscle, pale yellow nerve and pinkish brown ganglionic cells; nuclei intensely blue, liver cells well presented, and the different elements of pancreas brown (endocrine) and violet (exocrine).

KNOWER, H. McE. A rapid method for staining sections of the spinal cord and brain stem. Science, **72**, 172–3. 1930.

A method to demonstrate the microscopic anatomy and to correlate this with gross structure. It is an adaptation of Cole's "Rapid iron hematoxylin technique" (Sci. **44**, 452), modified for special material and thick sections, and is especially valuable for students. It may be used on old fixed material or fresh tissue hardened either immediately after dissection or by injection of formalin before removal from body.

Cut (free-hand), with a sharp, flat, thin razor, sections preferably 1 mm. or more thick, or frozen sections 100 μ thick.

From 95% alcohol (minimum 5 minutes) transfer to Cole's mordant (50% alcohol 20 cc., ferric chloride 1 g., glacial acetic acid 2 cc.) for at least 5 minutes, and then to alcohol. After prolonged mordanting staining is sometimes superfluous; but the following stain is recommended:

To 5 drops Cole's "stock hematoxylin" (absolute alcohol 20 cc., sodium bisulfite 0.2 g., dist. water 5 drops, light colored hematoxylin crystals, 1 g.) add 10 drops water and 1 drop ammonium hydroxide. After 30 seconds add 5 cc. 95% alcohol. Stain sections about 5 minutes and differentiate with 0.4% HCl. If necessary, remove sediment with cotton or lens paper. Rinse in slightly alkaline 95% alcohol after securing proper definition.

Study by strong reflected light. Examine with hand lens, binocular spectacles, or a low power (48 mm.) objective on a microscope.

LIENGME, A. Modificatio la méthode de coloration nucléaire en masse de Moret et Bassal. Bull d'Histol. Ap pl., **7**, 233–236, 1930.

This is a modification of the bulk nuclear stain of Boret and Bassal. It enables better penetration and renders the stain more uniform.

Fix pieces of tissue or whole embryos in any of the fixing fluids except osmic acid. Wash thoroly, especially after Bouin's solution. Transfer to 70 to 80% alcohol for 12 or more hours.

Stain in a mixture of equal parts of Boehmer's hematoxylin and Moret and Bassal's solution (3% aqu. sol. ferric chloride 2 cc., concentrated HCl 1 cc., 4% aqu. sol. copper acetate 1 cc., water 96 cc), for 24 hours to 4 days. This mixture can be used only once.

Differentiate in approximately 50% alcohol acidulated with HCl, (3 drops to 50 cc. alcohol), to remove the purplish color from the cytoplasm. Occasionally nuclei are overstained, in which case use dilute non-acidulated alcohol. Transfer to 70% alcohol for 12 to 24 hours. Counterstain in 0.5% alcoholic methyl-eosin according to Bujard, (Cahier de Technique micriscopique à l'usage des élèves du Laboratoire d'Histologie de Genève. Geneva, 1929), for 24 to 48 hours. Wash, dehydrate, embed as usual.

MACARTHUR, E. H. The behavior of sudan III when fed with carbohydrate. Science, **72**, 14. 1930.

Some carbohydrates mixed with sudan III may deposit colored fat in the body as stained fats do. Rats 6 to 8 weeks old or more were fed 4 to 5 days with either corn starch, rice starch, lactose, sucrose or dextrin mixed with sudan III (80 g. of the carbohydrate to 1 g. of sudan). Nothing except distilled water was added to that diet. On autopsy the dye was found deposited especially in the subcutaneous fat and in that surrounding the intestines and the testes. Urine and feces were also colored.

The question whether or not the dye fed with carbohydrates attaches itself to fat in its course thru the body or whether fat is synthesized from the carbohydrate fed and deposited as colored fat can not yet be answered.

SANNOMIYA, N. Über eine elektive Färbung der sogenannten Nebenkerne des Pankreas, nebst einigen Bemerkungenüber das Wesen derselben auf Grund dieser spezifischen Färbung. Folia Anat. Jap., **5**, 201–211. 1927.

The secondary nuclei of the pancreas are electively stained by the following method:

Fix in 10% HCl for 24 hours, running water 24 hours. Embed in celloidin and cut in the usual manner.

Stain in a 1% solution of aqueous acid fuchsin 3 minutes or longer; wash in distilled water.

Differentiate in a saturated solution of picric acid 3 to 4 minutes; rinse in distilled water.

Transfer to a 1% solution of phosphomolybdic acid 1 to 2 minutes; rinse thoroly in distilled water.

Dehydrate, clear and mount.

The secondary nuclei are brilliant red, all other elements gray to yellow. Counterstaining with light green or staining beforehand with Böhmer's hematoxylin are very useful for topographic study.

An alternate procedure is:

Place sections in 1% aqueous solution of phosphomolybdic acid for 1 to 3 minutes and rinse.

Stain 1 to 2 minutes in the following: 0.5 g. anilin blue, 2 g. orange G, 100 cc. distilled water; rinse. (Dyes probably from Grübler, but source not named.)

Differentiate in 96% alcohol.

Dehydrate; pass thru xylol to balsam.

Secondary nuclei are yellow or yellow-brown, while all other elements are quite different. Connective tissue is blue, cytoplasm and nuclear bodies dark gray.

SATO, K. On the staining capacity of the nucleolus and its physiological significance. Folia Anat. Jap., 6, 717–736. 1928.

Eggs of *Cristaria plicata* were chosen for the study of nucleoli. Observations concerning histologic technic will be mentioned here.

The nucleolus is composed of a deeper stained plasmosome and lighter stained karyosome. The difference is demonstrated by any stain. The plasmosome is the denser part from which comes the karyosome and then the loosest chromatin. They are similar in chemical nature. Plasmosomes resist the action of acid and alkaline solutions most strongly. Karyosomes and accessory nuclei are next to it in this respect, while chromatin is most sensitive to these chemicals. The so-called acidophile part (plasmosome) is always more deeply stained than the so-called basophile part (karyosome) by any dyestuff, no matter whether acid or basic. When a mixture of two dyes is used the acidophile part is stained by the one of greater diffusibility. The degree of infiltration of the stain varies according to the time of application. Deep staining and light staining seem more appropriate terms than acidophile and basophile elements. Alcohol, acetone, Carnoy's, Zenker's fluids bring out the double nature of the nucleolus. Formalin, Schaffer's, Orth's solutions, chromates, and boiling destroy it.

SATO, K. On the influence of electric polarization upon the stainability of nerve. Folia Anat. Jap., 7, 33–43. 1929.

Bethe's method of electric polarization was used to study the stainability of nerve (sciatic, frog). The stainability is generally weak in the cathodic portion. Reaction of the dye, unequal distribution of fibrillar acid, the nature of the electrode make no difference. The density of the tissue at both poles is very important from the standpoint of the physical theory of staining. At the deep staining anodic portion not only the axis cylinders, but also connective tissue and nuclei are condensed, at the less staining cathodic portion they are loosened. Generally speaking, loose tissue which is fluid can scarcely hold any stain, while those which are compact and poor in fluid do not loose the stain already imbibed. The difference in density of the tissue at both poles is caused by some vital phenomenon during electrification. The latter is absent in narcotized, poisoned or heated nerve. The difference of stainability is influenced by the intensity and duration of the electric current, by the nature of the medium in which the nerve preparation is kept, and by temperature. During the passage thru the tissue K-ions go faster to the cathode than Ca ions. The ratio K/Ca becomes greater than normal, and it decreases at the anode. This may explain why the tissue becomes loose at the cathodic and dense at the anodic portion. K enhances the cathodic effect and makes the difference of density and stainability of the tissue more pronounced, while Ca inhibits the effect and minimizes the difference.

Tschernyachinsky, A. Eine Vervollkommnung des Paraffin–Zelloidin–Verfahrens. Zts. Wis. Mikr., 47, 200–201. 1930.

This is an improvement of a previously described celloidin-paraffin method (Zts. Wis. Mikr., 42, 443.) The introduction of clove oil enables better cutting. Various differential staining is possible.

Transfer material from celloidin to 3 to 4% celloidin in clove oil (to a 6 to 8% celloidin solution in ether-alcohol add an equal quantity of clove oil), for 2 to 24 hours;

Place in chloroform ½ hour;

Transfer to chloroform-paraffin in oven, and leave until material sinks, (½ to 1 hour);

Introduce into pure paraffin.

The method saves time when used following Cajal's impregnation, by the following procedure:

Transfer section serially from microtome to slides;
Dissolve paraffin with xylol;
Flatten out by pressing with filter paper;
Mount in Canada balsam.

The author thinks that silver impregnation should not fade within a year's time and recommends it for various critical studies. Gold impregnation should not be used.

WALTER, L. Neuartige Anwendung des Pasinischen Farbengemisches zur feineren Untersuchung des Bindegewebes. Zts. Wis. Mikr., **46**, 457–463. 1930.

This is a modification of Pasini's stain for better differentiation of connective tissue.

Fix in sublimate, formalin, Carnoy's solution or best of all in Heidenhain's "Susa" mixture. (See Romeis' Taschenbuch, 12 Aufl. p. 80.)

Embed as desired, but preferably by the celloidin-paraffin method of Apathy (Zts. Wis. Mikr., **29**, 449–515. 1913).

Cut sections 3 μ thick. Dissolve paraffin with benzol and celloidin with ether alcohol, and pass thru alcohols to water. Use iodine-alcohol and sodium thiosulfate for material fixed with sublimate.

Mordant in 2.5% iron alum 24 hours for sections 2 to 4μ thick and shorter for heavier, rinse in distilled water.

Transfer to Pasini's solution (Unna's anilin-blue-orcein, 10 drops; 2% eosin B in 50% alcohol, 12 drops; saturated aqueous acid fuchsin, 1 drop; neutral glycerine, 5 drops.) Source of stains not given.

Transfer to 96% alcohol and agitate as long as color clouds are given off (about 1 minute), then to 100% for 1 minute. Blot; pass thru xylol to balsam.

Results: Collagen fibers deep blue, cytoplasm carmin red. Connective tissue and wandering cells, smooth and striated muscle, epithelia are all well defined. Epithelial cells, centrioles, basal bodies, nuclear structure are brilliant red. Secretory bodies vary according to their nature. The slime of the goblet cells is azure blue. Erythrocytes yellowish red and therefore show up brilliantly in the blue connective tissue.

The method is especially valuable in histologic research. The stain keeps well both in solution and in the preparations.

PROGRESS IN THE STANDARDIZATION OF STAINS

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the July number of this journal.

Stains Certified May 15 to August 15, 1930*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Orange G	NO 3	85%	As histological counterstain	May 16, 1930
Nigrosin, W. S.	NNi 4	——	As bacteriological background stain	June 1, 1930
Wright stain	NWr 6	——	As blood stain	June 19, 1930
Acid fuchsin	NR 8	65%	As histological counterstain, and in the Andrade indicator	Aug. 13, 1930
Crystal violet	NC 10	91%	As cytological and bacteriological stain	Aug. 13, 1930
Wright stain tablets	BWr 1	——	As blood stain	Aug. 13, 1930

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

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